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Research Article

# Methyl jasmonate enhanced production of rosmarinic acid in cell cultures of *Satureja khuzistanica* in a bioreactor

The growing interest in rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid, is due to its biological activities, which include cognitive-enhancing effects, slowing the development of Alzheimer's disease, cancer chemoprotection, and anti-inflammatory activity. Inspired by the challenge of meeting the growing demand for this plant secondary metabolite, we developed a biotechnological platform based on cell suspension cultures of *Satureja khuzistanica*. The high amounts of RA produced by this system accumulated mainly inside the cells. To further improve production, two elicitors, 100 $\mu$M methyl jasmonate (MeJA) and 40 mM cyclodextrin (CD), were tested, separately and together. MeJA increased RA productivity more than 3-fold, the elicited cultures achieving an RA production of 3.9 g $L^{-1}$ without affecting biomass productivity. CD did not have a clear effect on RA production, and under the combined treatment of MeJA + CD only a small amount of RA was released to the medium. When the cell culture was transferred from a shake flask to a wave-mixed bioreactor, a maximum RA production of 3.1 g $L^{-1}$ and biomass productivity of 18.7 g $L^{-1}$ $d^{-1}$ was achieved under MeJA elicitation, demonstrating the suitability of *S. khuzistanica* cell suspensions for the biotechnological production of this bioactive plant secondary metabolite.

**Keywords:** Cyclodextrin / Methyl jasmonate / Plant cell cultures / Rosmarinic acid / Satureja khuzistanica

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## 1 Introduction

Rosmarinic acid (RA), an ester of caffeic acid and (R)-(+)-3-(3,4-dihydroxyphenyl) lactic acid, is a bioactive component of several medicinal plant species [1]. According to current knowledge, RA is found in 39 plant families, including the Lamiaceae and Boraginaceae, but also several species of the Choranthaceae or Blechnaceae, as well as some orders of the monocotyledonous plants, and the rosids and asterids within the eudicotyledoneae [2, 3, and references therein]. A newly reported natural rich source of RA is *Satureja khuzistanica* Jamzad of the Lamiaceae, an endemic plant of Iran, whose RA contents in methanol (MeOH) extracts range from 0.59 to 1.81% [3].

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**Abbreviations: CD**, cyclodextrin; **CDW**, cell dry weight; **CFW**, cell fresh weight; **MeJA**, methyl jasmonate; **pcv**, packed cell volume; **RA**, rosmarinic acid; **WV**, working volume

The well-studied bioactivity of RA [4] includes antimicrobial, anti-inflammatory, and antioxidant activities, and it is used to treat peptic ulcers, arthritis, cataracts, cancer, rheumatoid arthritis, and bronchial asthma, among other illnesses [5]. RA is also applied as a preservative of fresh seafood, especially in Japan [6]. One of the main properties of RA is its antioxidant capacity, which is likely due to an ability to stabilize membranes and prevent free radical movement [7]. Awareness of the potential benefits of RA as a pharmaceutical or dietary supplement for human health is growing. Among its most promising biological activities are cognitive-enhancing and cardioprotective effects, cancer chemoprevention properties, and a potential use in the treatment of Alzheimer's disease [8-9].

Many RA-producing biotechnological platforms have been established, including shoots [10]; cell suspensions [5]; and hairy root cultures [11] of numerous species of the Boraginaceae, Anthocerotaceae, and Lamiaceae (reviewed in Bulgakov et al. [4]), including *Satureja khuzistanica* [12, 13]. Several elicitor treatments have been applied to enhance RA yields, including fungal elicitors, cuprum ions, silver ions, salicylic acid, or methyl jasmonate (MeJA) [4,9]. Another approach is to facilitate

downstream processes using permeabilizing agents such as DMSO [6]. In this context, cyclodextrins (CDs), which are cyclic polymers of D-glucose linked by a-1,4-glycosidic bonds [14], have been tested in plant cell cultures for the production of bioactive secondary metabolites [15]. Unlike other permeabilizing agents, in cell cultures of some plant species, CD can trigger plant secondary metabolism by acting as true elicitors, inducing defense responses as well as promoting the release of target compounds to the culture medium [16, 17].

The last step in the process development of a biotechnological production system is scaling up to bioreactor level. Reusable and disposable bioreactors have been successfully applied [18, 19], but to date, only the former have been used for the production of RA with plant cells. Ulrich et al. [20] developed a two-stage RA production system with a *Coleus blumei* cell suspension culture in an airlift bioreactor. Su and Lei [21] used the perfusion mode in a stirred tank bioreactor for RA production with an *Anchusa officinalis* suspension culture, reporting an optimum 30% of air saturation [22]. More recently, Pavlov et al. [23], working with a 3 L stirred bioreactor, demonstrated that optimum settings for RA production in *Lavandula vera* cell suspensions were a $dO_2$ of 30%, 300 rpm, and a temperature of 28°C. Zhong et al. [24] investigated the kinetics of *Salvia miltiorrhiza* cell cultures in stirred bioreactors with different impeller sizes, showing that a large turbine impeller reactor was preferable for cell growth and RA production.

In the current work, based on a callus-derived *S. khuzistanica* cell line, we developed and optimized a biotechnological platform for RA production using the elicitor MeJA. In optimal conditions, the system achieved a productivity of 4 g $L^{-1}$ of RA, one of the highest yields achieved to date for a plant secondary metabolite. In the same system, the effect of the permeabilizing agent CD was also tested.

## 2 Materials and methods

### 2.1 Plant cell cultures

To obtain a homogenous cell suspension of *S. khuzistanica*, 10 g calli (initiated and routinely subcultured as described by Sahraroo et al. [12]) were transferred to a 1 L Erlenmeyer flask with a working volume (WV) of 200 mL of optimized Gamborg's B5 medium [25], which was supplemented with 30 g $L^{-1}$ sucrose, 20 mg $L^{-1}$ L-glutamine, 200 mg $L^{-1}$ casein hydrolysate, 5 mg $L^{-1}$ benzyl-aminopurine, and 1 mg $L^{-1}$ indole-3-butyric acid (all reagents were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise specified). The culture conditions for all the experiments performed on a small-scale (Erlenmeyer flasks) were as follows: a temperature of 25°C, 110 rpm shaking frequency, 25 mm shaking diameter, and darkness.

### 2.2 Elicitor experiment

For this experiment, 125 mL flasks with a WV of 25 mL and an inoculum packed cell volume (pcv) of 10% (1-wk-old) *S. khuzistanica* cell suspension were utilized. After 7 days of culture, randomly methylated-$\beta$-CD) at a final concentration of 40 mM was added to the flasks, and after 2 days MeJA at a final concentration of 100 $\mu$M was added to the cultures previously supplemented with CD and also to control cultures without CD. As MeJA was dissolved in ethanol (EtOH), 50 $\mu$L of EtOH was also added to the cultures (mock conditions). The culture period consisted of 21 days and samples were harvested at 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21 days of growth in order to determine their pcv, cell fresh weight (CFW), cell dry weight (CDW), pH, conductivity, and RA production.

### 2.3 Transfer to a benchtop bioreactor

A disposable mechanically driven bioreactor (BIOSTAT RM 20/50 from Sartorius Stedim Biotech) was used to study the RA production in the *S. khuzistanica* cell line on a benchtop scale. Two different sets of experiments were performed at bioreactor level: under control or optimized elicitation (MeJA) conditions, based on the results we obtained in the small-scale experiments (shake flasks from Thermo Fisher Scientific). The culture bags were filled with optimized B5 medium and a *S. khuzistanica* cell suspension with a viability of >95% to reach a pcv of 10% in the CultiBag. For the elicitor experiments, at day 11 a sterile solution of MeJA was added to the culture at a final concentration of 100 $\mu$M. In the BIOSTAT RM, culture bags of 2 L (1 L WV) were used. The experiments lasted 21 days and were carried out in batch mode in the dark at 25°C. Wave-mixed culture bags were operated at a rocking angle of 6° and an aeration rate of 0.1 vvm. In order to avoid mass transfer limitations resulting from increasing biomass and viscosity, the rocker rate of the BIOSTAT RM was increased from 20 to 30 rpm during all cultivations. The initial biomass concentration was 45 g CFW $L^{-1}$.

### 2.4 In-process controls

Samples were harvested according to the sampling program and checked for viability, pcv, CFW, CDW, pH, and conductivity as described previously [26]. Briefly, in order to calculate the percentage of living cells, samples were colored with Evans Blue and observed under the microscope (200×). For the pcv, 10 mL of cell suspension was transferred to 15 mL Falcon tubes and centrifuged at 4000 rpm for 10 min. The pcv was read in the tube and the pellet was transferred to a filtration unit, and connected to a vacuum pump for 3 min. After determination of the CFW the samples were kept in the freezer at −20°C. Frozen biomass was freeze-dried for 24 h and the CDW of each sample was registered. Additionally, supernatants were collected and stored in the freezer (−20°C) until the end of the experiment. Conductivity and pH were analyzed in the supernatant using a MC266 conductivity meter and Five Easy pH meter.

### 2.5 RA extraction and quantification

Extraction and quantification of RA from lyophilized cells and supernatants was performed following the protocol described by Georgiev et al.[27 with some modifications.

#### 2.5.1 Extraction from freeze-dried biomass

Twenty milligram dried cells were mixed with 9 mL methanol. After 2 min in vortex, the extract was incubated for 20 min in an ultrasonic bath, and then centrifuged (4000 rpm). The supernatant was dried in a rotary evaporator under reduced pressure, and the residue was dissolved in 1.5 mL of methanol. Then the samples were passed through a 0.2 $\mu$m filter before HPLC analyses.

#### 2.5.2 Extraction from supernatant

Ten milliliter of filtered culture medium was frozen and lyophilized. Dry extracts were dissolved in 5 mL of methanol (for HPLC), refrigerated for 24 h at 4° C, and then filtered (0.45 or 0.2 $\mu$m) and injected into the HPLC system.

#### 2.5.3 HPLC analyses of RA

For quantitative determination of RA, we followed the protocol described by Sahraroo et al. [12]. An aliquot of 20 $\mu$L of the filtrate samples was injected into the HPLC for analysis. Three hundred thirty nanometers was selected as the wavelength for UV detection. The HPLC column was a Spherisorb ODS-2 (5 $\mu$m) reverse phase 4.6 × 250 mm$^2$ (Waters Corporation, Mildford, U.S.A.). Elution was carried out at a flow rate of 1.0 mL min$^{-1}$ at 25°C. Two mobile phases, A and B, were used. Mobile phase A was 0.1% v/v formic acid solution in water, while mobile phase B was acetonitrile. A ratio of 88% A and 12% B was applied in the first 30 min. After 30 min, a ratio of 80% A and 20% B was used for the next 15 min. Finally, 70% A and 30% B were used after 45 min for an additional 15 min. The chromatographic peak of RA was confirmed by comparing its ESI–MS spectra and retention time with that of the reference standard (see Supporting Information). Working standard solutions were injected into the HPLC and peak area responses obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

### 2.6 Statistics

Statistical analysis was performed with Excel software. All data are the average of three determinations ± SD. A multifactorial analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used for statistical comparisons. A $p$-value of $<0.05$ was assumed for significant differences.

## 3 Results

### 3.1 Elicitor effects on growth

The effects of the elicitor MeJA were tested at a concentration of 100 $\mu$M and the elicitor/permeabilizing agent randomly methylated-$\beta$-CD at a final concentration of 40 mM. Both elicitors have been previously used to increase plant secondary metabolism in other plant cell cultures and in most cases, the aforementioned concentrations have been determined as optimal for inducing the accumulation of the target compound [17, 28]. An inoculum pcv of 10% (1-wk-old) was prepared to optimize the growth capacity of the *S. khuzistanica* cell suspension.

The time course of growth measured as CFW shows that after a short lag phase of 1–2 days, the cultures entered the exponential growth phase, which lasted until day 7 (Fig. 1A), when the inoculum doubling time was approximately 2.5 days. Thereafter, a slight increase of CFW was achieved and at day 11 the culture entered the stationary growth phase, reaching a final biomass of 309.5 g L$^{-1}$ in control conditions (Fig. 1A). Elicitor treatments only affected the growth capacity at the end of the culture (days 18–21), when a small increase of CFW was observed in CD-treated cells, while the addition of MeJA, alone or together with CD, slightly reduced the biomass concentration.

The CDW time course was very similar, although the stationary phase began earlier, at day 7, differing from the CFW curves (see above). This difference could be explained by the cells absorbing water without gaining DW from days 7 to 11. A significant decrease of CDW occurred in MeJA-elicited cells (Fig. 1B). This negative effect was not observed in the cell culture treated with CD + MeJA, which suggests a positive effect of CD on the CDW of MeJA-elicited *S. khuzistanica* cell suspensions.

The conductivity decreased during the first week of the culture due to the consumption of the medium salts. From days 7–9, the conductivity of the CD experiments increased significantly ($p < 0.05$), probably due to a direct effect of CD on cell permeability and the release of mineral ions from inside the cells (Fig. 1C). After this period, conductivity decreased continuously until day 16, when it increased slightly, especially in control and CD + MeJA treated cell cultures. This increased conductivity at the end of culture period could be due to cell death and the consequent release of ions from inside the cells. Treatment with MeJA did not significantly affect cell conductivity.

The initial pH of the suspension culture was 5.7, which dropped to 5.2 at day 2, probably due to massive ammonium uptake (Fig. 1D). After day 2, the pH started to rise due to nitrate uptake. Elicitation did not significantly affect the pH of the cell cultures and CD alone caused a slight decrease, especially at the end of the culture period.

### 3.2 Effects of the elicitors on RA production

In control conditions, RA production in the cells increased from the beginning of the experiment until the end, achieving a final RA content of 84 mg g$^{-1}$ DW (1.2 g L$^{-1}$) at day 21 (Fig. 2A and B). MeJA treatment dramatically increased ($p < 0.05$) the production of intracellular RA in the *S. khuzistanica* cell cultures, and after 1 wk of elicitation (day 16) RA content peaked at 245 mg g$^{-1}$ (3.86 g L$^{-1}$), which represents a significant 3.2-fold increase compared with the control cells at the same time point (Fig. 2A and B). The effect of the permeabilizing agent CD was only moderate, provoking an increase ($p < 0.05$) of RA contents inside the target cells only after 11 days of treatment (day 18; Fig. 2A). The combined MeJA + CD treatment achieved a much lower intracellular RA production than MeJA alone (Fig. 2B).

CD has been used not only as an intracellular elicitor in plant cell cultures, but also as a permeabilizing agent [17].

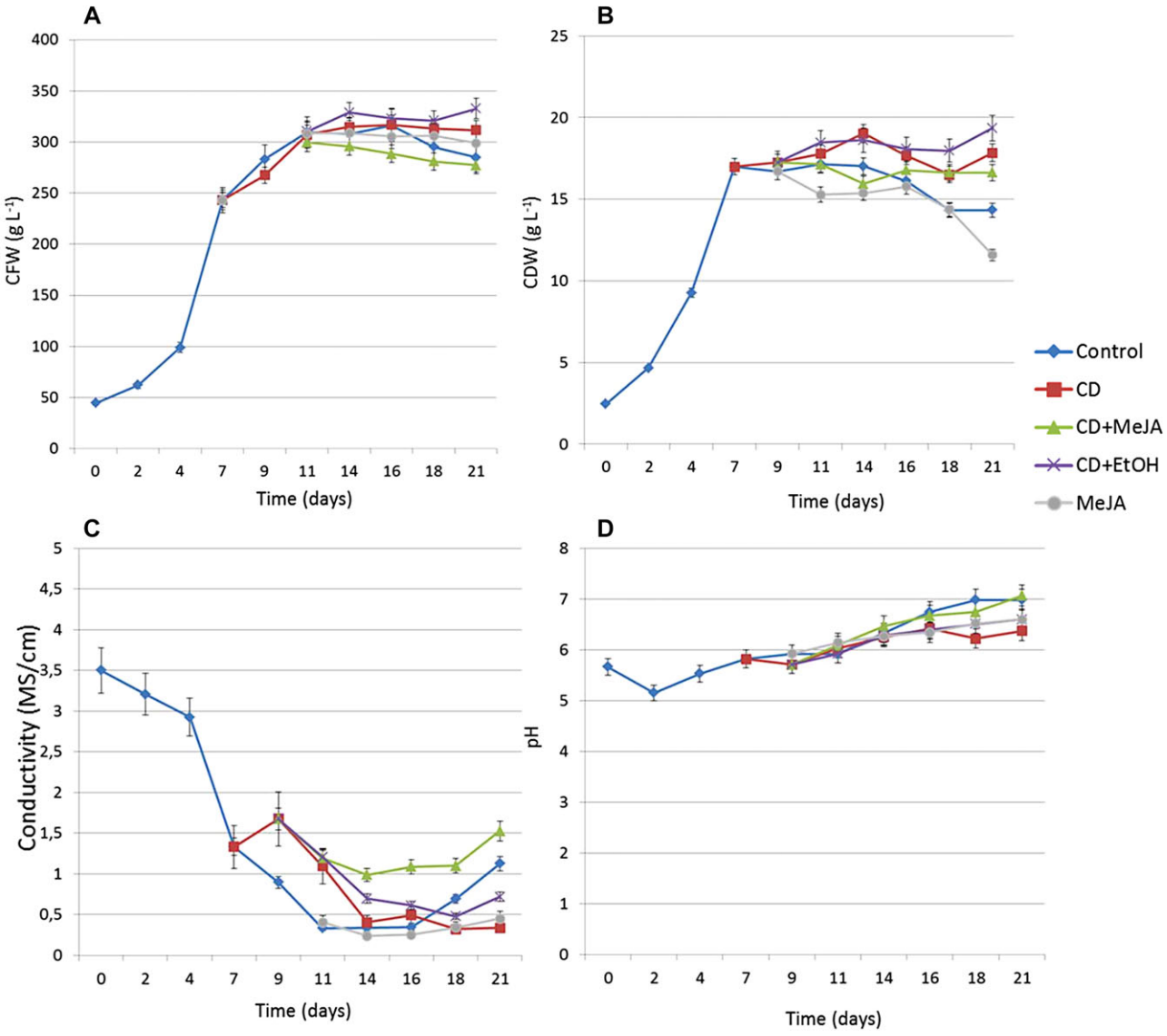


**Figure 1.** Time course of the growth measured as (A) CFW and (B) CDW, (C) conductivity, and (D) pH of *Satureja khuzistanica* cell cultures in shake flasks. Each value is the average of three biological replicates ± SE.

Figure 2B shows the total RA production of the cultures. RA accumulated mainly inside the cells and only small amounts were released to the cell medium. Toward the end of the experiment, the CD treatment seemed to have slightly increased the release of RA from cells to the culture medium. The addition of MeJA alone did not affect the RA excretion, with only traces being detected in the culture media. In contrast, MeJA slightly increased the extracellular contents of RA in the CD-treated cell cultures, especially at day 18 (0.38 g $L^{-1}$), probably due to its stimulatory effect on RA production (Fig. 2B).

It is worthwhile mentioning that at the end of the culture period, CD-treated cells remained pale yellow, while the culture medium became dark, whereas in control cultures, the cells were much darker, while the culture medium remained a lighter color (Fig. 3). This effect could be due to the release of phenolic compounds from the cells to the culture medium and therefore could confirm the role of CD as a permeabilizing agent in our cultures. However, further analyses would be necessary to confirm this.

### 3.3 Transfer to a benchtop bioreactor

In order to transfer the process to a benchtop bioreactor in conditions optimized for RA production, a wave-mixed BIOSTAT RM was utilized. As mentioned previously, the bioreactor was run in batch mode and after 11 days, the cell culture was treated with 100 $\mu$M of MeJA. The obtained results were referred to biomass, and RA production was compared with levels obtained in control conditions (untreated cell cultures).

An inoculum pcv of 10% (1-wk-old) was prepared to optimize the growth capacity of the *S. khuzistanica* cell suspension in the wave-mixed bioreactor and samples were taken every 2–3 days

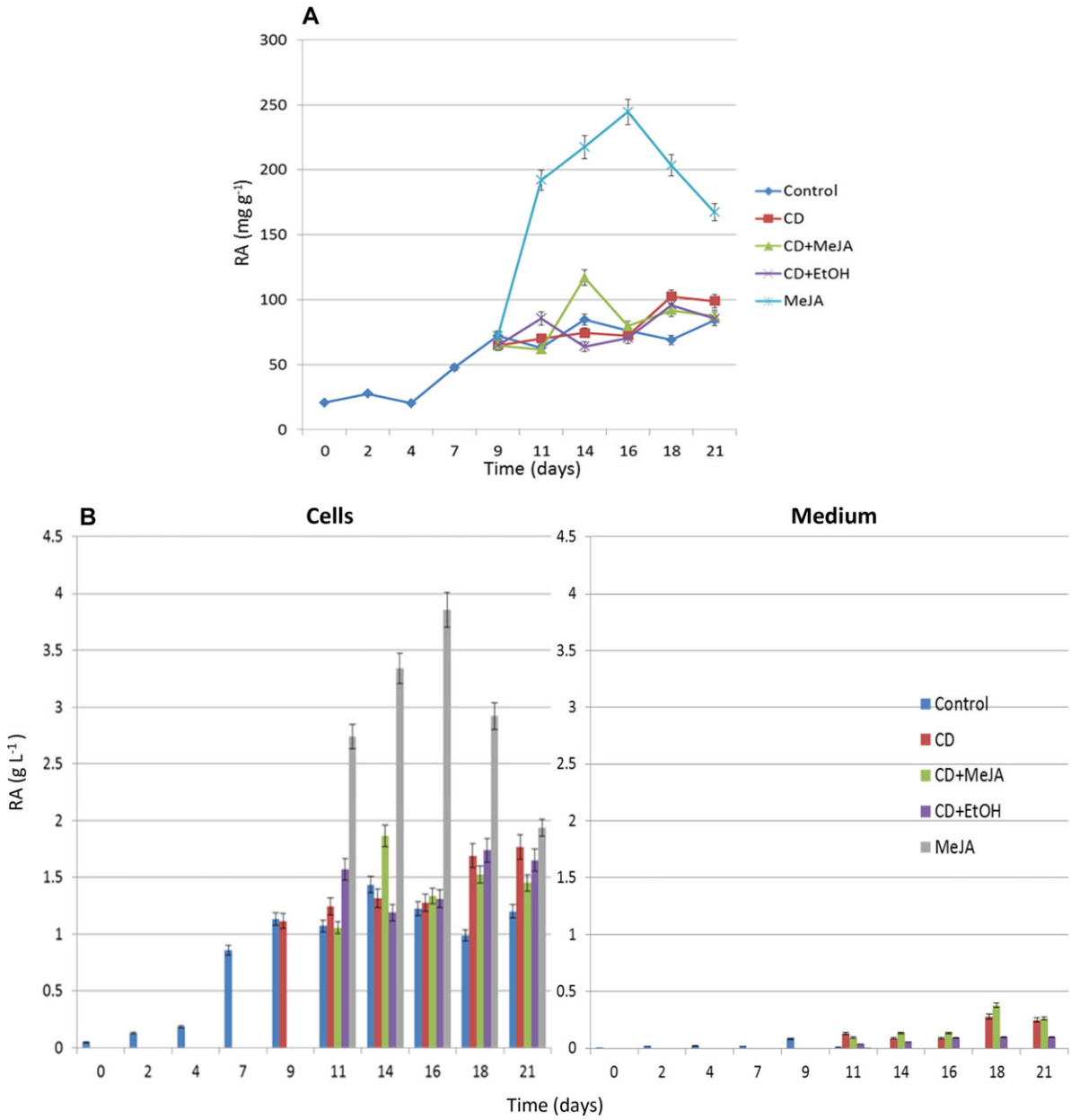


**Figure 2.** Time course of the rosmaric acid production of *Satureja khuzistanica* cell cultures in shake flasks expressed as (A) mg g $DW^{-1}$ or (B) g $L^{-1}$. Each value is the average of three biological replicates ± SE.

during a culture period of 21 days. The time course of fresh biomass shows that after a lag phase of 2 days, CFW increased significantly, reaching a maximum at day 14, when the culture began its stationary phase (Fig. 4A). The results also show the inhibitory effect of MeJA, mainly at the end of the culture period, when the CFW decreased from 363 g $L^{-1}$ at day 11 to 316 g $L^{-1}$ at day 14.

The CDW of the cultures showed a similar growth course. MeJA treatment provoked a dramatic decrease in the CDW (Fig. 4A), as in the CFW. The time course of pH and conductivity in the bioreactor cultures (Fig. 4B) was very similar to the shake flasks.

RA production in the *S. khuzistanica* cell suspension measured as gram per liter increased continuously over the culture period in control conditions, reaching a final production of 3.1 g $L^{-1}$ (Fig. 4C), which represents an RA productivity of 0.15 g $L^{-1}$ $day^{-1}$ (Table 1). The addition of MeJA at day 11 to cell suspensions cultured in the wave-mixed bioreactor increased RA production 1.2-fold, reaching 3.6 g $L^{-1}$ at the end of the culture period (Fig. 4C). When these results are compared with those obtained in shake flasks (Fig. 2), the suitability of the wave-mixed bioreactor for the *S. khuzistanica* suspension cell-based RA production is clearly demonstrated. In control conditions, RA production in the bioreactor system (3.1 g $L^{-1}$) after 21

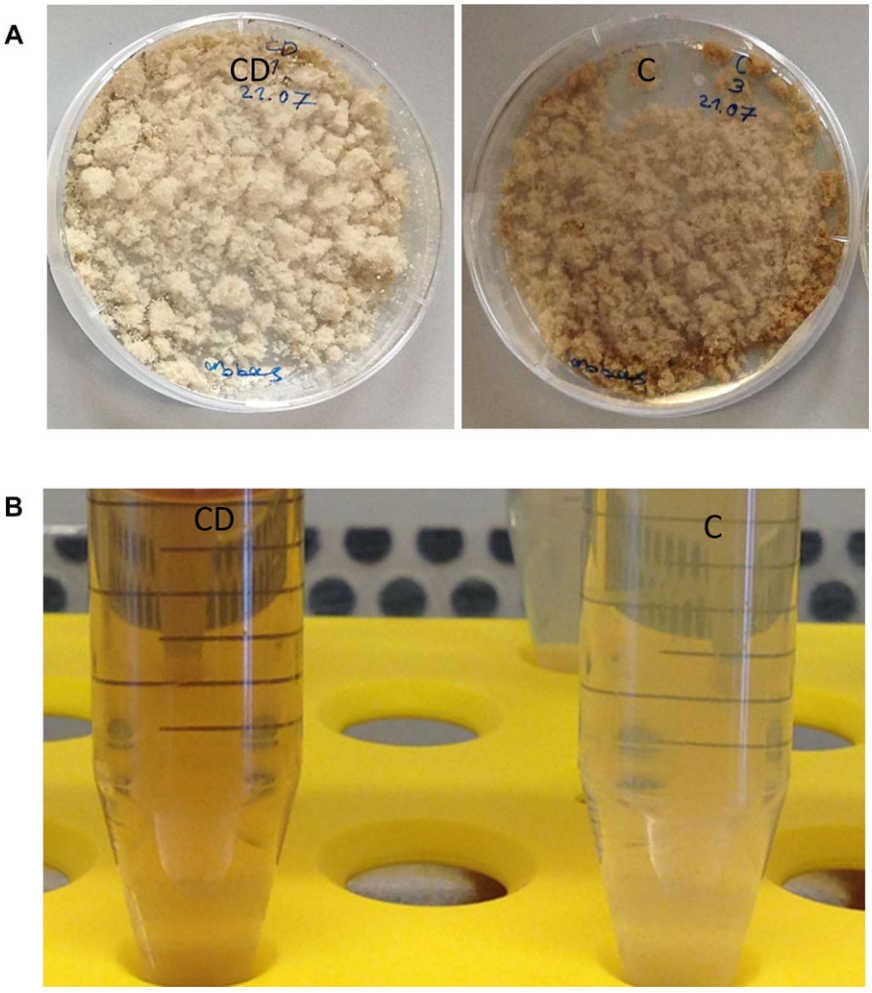


**Figure 3.** Comparative aspect of (A) cell biomass and (B) culture media of the control (C) and after 6 days of the treatment with CD.

days of culture was 2.6-times higher than in shake flasks on the same day (1.2 g $L^{-1}$). On the other hand, the results also show that whereas RA peaked at day 16 in the elicited small-scale cultures (3.9 g $L^{-1}$), at bioreactor level the maximum production under elicitation was achieved at the end of the culture period (3.6 g $L^{-1}$) at day 21. Consequently, at the end of the experiment, the RA productivity of the system was 0.17 g $L^{-1}$ $day^{-1}$ in the wave-mixed bioreactor, compared to 0.09 g $L^{-1}$ $day^{-1}$ in the shake flasks (Table 1).

## 4 Discussion

The productivity of a biotechnological system depends not only on the capacity of the plant cells to produce and accumulate the target compounds, but also on its capacity to produce cell biomass [29]. In this study, the *S. khuzistanica* cell line showed a typical growth curve with a short lag phase of 1–2 days and an exponential phase between 7 and 11 days, followed by a stationary and dying phase until the end of the culture period at day 21 (Fig. 1). During the growth period, the high capacity of this optimized system to generate cell biomass was demonstrated by a 5-fold increase on the biomass previously obtained with the same cell line in nonoptimized conditions [12].

In general, elicitor treatments did not significantly affect the CFW and CDW of the cultures, with the exception of 100 $\mu$M MeJA, which significantly reduced the CDW at the end of the experiment. The growth inhibition caused by 100 $\mu$M MeJA in *S. khuzistanica* cell cultures has also been reported in cell cultures of other RA-producing plant species [30–32]. However, in our study, this negative effect of MeJA on biomass (see Fig. 1) was reversed by the addition of CD. Although this compound has not been tested in other RA-producing cell lines, Sabater-Jara et al. [33] reported a positive effect of CD on the growth capacity of *Taxus media* cell cultures.

The enhancing effect of MeJA on RA contents in the *S. khuzistanica* cell biomass was demonstrated. Jasmonic acid and its derivatives are involved in the defense signals of plants [34]. For example, hydroxyethyl jasmonate induces $H_2O_2$ (oxidative bursts), lipoxygenase activity, and intracellular jasmonic acid synthesis, as well the expression of several genes involved directly in taxane biosynthesis in *Taxus* spp. cell cultures [34,35]. In this work, after a week of MeJA treatment, the *Satureja* cells accumulated up to 3.2-fold more RA than the control cells, achieving an approximate RA concentration of 25% CDW and

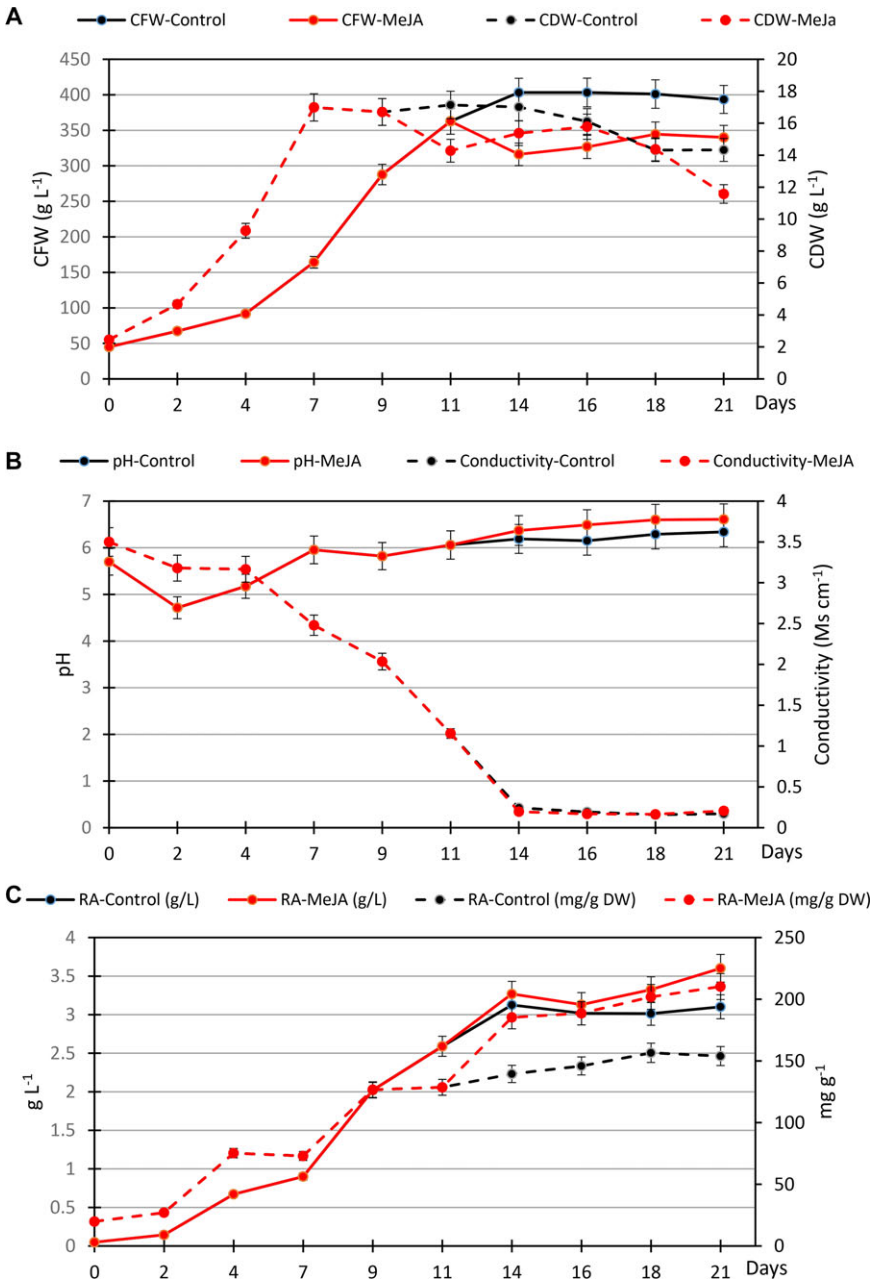


**Figure 4.** (A) Time course of the growth measured as CFW (g $L^{-1}$) and CDW (g $L^{-1}$), (B) pH and conductivity (mS $cm^{-1}$), and (C) RA production (expressed as g $L^{-1}$ and mg $g^{-1}$ DW) of *Satureja khuzistanica* cell cultures in the wave-mixed bioreactor under control and MeJA elicitation conditions. Each value is the average of three replicates ± SE.

3.86 g $L^{-1}$ (Fig. 2), one of the highest RA yields reported in plant in vitro cultures so far. Positive effects on RA accumulation in MeJA-elicited cell cultures have been reported previously [4]. As in taxane biosynthesis [35], recently, Kim et al. [36] described a correlation between RA production and the expression level of several genes involved in RA biosynthesis in MeJA-elicited *Agastache rugosa* cell cultures. Such a metabolic effect induced by MeJA would explain the improved RA production in the *S. khuzistanica* cultures, although this hypothesis has to be further studied.

Although very high amounts of RA were produced by the *S. khuzistanica* cell cultures, its isolation requires extraction from the dry material. RA-producing cell cultures mainly accumulate RA intracellularly in vacuoles [37, 38]. Thus, in order to facilitate RA extraction, Park and Martinez [39, 40] permeabilized *C. blumei* cell suspensions with 0.1% of DMSO, but unfortunately the RA released to the culture medium was rapidly metabolized by peroxidases [41].

Regarding the permeabilizing effect of the CD treatment, only small amounts of RA were found in the medium at the end of the culture period, being higher with the combined CD + MeJA treatment. This low effect of CD in the *S. khuzistanica* cell suspensions contrasts with previous reports on *Vitis vinifera* or *Taxus* sp. cell cultures, in which CD simultaneously acted as strong elicitors and permeabilizing agents [17, 33]. Nevertheless, after the CD treatment, our cells remained a pale

**Table 1.** Comparative study of the growth capacity and RA production of the *Satureja khuzistanica* cell suspensions in the both cultured system assayed, shake flasks, and BioSTAT® CultiBag RM

| Cultivation system | Treatment | Growth rate $\mu_{max}$ ($d^{-1}$) | Doubling time $t_d$ (d) | Biomass productivity $BMP_{CFW}$ ($g\,L^{-1}\,d^{-1}$) | RA productivity RAP ($g\,L^{-1}\,d^{-1}$) |
|---|---|---|---|---|---|
| Shake flasks | Control | 0.12 | 5.9 | 13.6 | 0.06 |
| | MeJA | 0.11 | 6.4 | 14.8 | 0.24 |
| | CD | 0.12 | 5.7 | 13.2 | 0.09 |
| | CD + MeJA | 0.12 | 5.8 | 14.2 | 0.14 |
| Bioreactor | Control | 0.16 | 4.4 | 18.7 | 0.15 |
| | MeJA | 0.14 | 5 | 16.1 | 0.17 |

$\mu_{max}$ = maximum-specific growth rate: $(\ln[X]_{DW2}\text{-}\ln[x]_{DW1}/(t_2 - t_1)$; $t_d$ = doubling time: Ln2/$\mu_{max}$; $BMP_{CFW}$ = biomass productivity: CFW/$t$; RAP = rosmarinic acid productivity: RA production ($g\,L^{-1}$)/$t$.

yellow color, while the culture medium turned dark, in contrast with the darker cells and lighter medium of the control cultures (Fig. 3). This effect could be due to the release of phenolic compounds from the cells to the culture medium, which would confirm the role of CD as permeabilizing agents. This fact, together with the low amount of RA found in the culture medium, points more to a degradation of RA outside the cells by peroxidase enzymes than "inactivity" of CD in this cell system. As mentioned above, and according to Szabo et al. [41], RA is rapidly metabolized outside the cells by the high apoplastic peroxidase activity.

Cell suspensions of several plant species have been used for the biotechnological production of RA but an industrial production of this compound has not been achieved so far [4]. Production of RA in bioreactors has been reported previously [9 and references therein], but not in disposable bioreactors. For example, cell suspensions of *L. vera* cultured in a stirred bioreactor in optimal conditions (50% air saturation, 400 rpm) achieved a maximum RA production of 3.5 g $L^{-1}$ [42], but the production decreased to 1.8 g $L^{-1}$ at an agitation rate of 300 rpm and 30% dissolved oxigen (DO) [23], thus confirming the importance of the culture conditions in reaching a high RA production.

When a cell culture has to be transferred to a bioreactor, several factors need to be taken into account, particularly the shear stress and $O_2$ transport efficiency [43]. Wave bioreactors constitute a very suitable type of disposable bioreactor due to their low cost and their efficiency in obtaining high levels of cell biomass as well as secondary metabolite production [19].

BIOSTAT RM 20/50, a disposable wave bioreactor, has been successfully used for the culture of cell suspensions of several plant species, including *V. vinifera*, *Malus domestica*, *Nicotiana tabacum*, *Taxus baccata*, and *Hordeum vulgare* (Eibl et al. [44] and references therein). Computational fluid dynamics (CFD) simulations showed a more homogeneous energy dissipation and shear stress pattern in this bioreactor than in stirred reusable bioreactors [45], which is probably why wave-mixed bioreactors may be superior for producing plant cell biomass. In this work, in control conditions, the biomass productivity ($BMP_{CFW}$) of the system was 18.7 g $L^{-1}$ $d^{-1}$ with an RA productivity of 0.15 g $L^{-1}$ $d^{-1}$, which is 1.4- and 2.5-fold higher, respectively, than that achieved in shake flasks (Table 1). Recently, Lehmann et al. [19], using the BIOSTAT RM with a transgenic tobacco cell line carrying the geraniol synthase gene of *Valeriana officinales* (VoGES), reported a $BMP_{CFW}$ of 10 g $L^{-1}$ $d^{-1}$ that produced up to 26 $\mu$g $g^{-1}$ FW of geraniol.

Under optimal elicitation conditions (100 $\mu$M MeJA), RA production in *S. khuzistanica* cell suspensions cultured in the BIOSTAT RM increased significantly, reaching 3.6 g $L^{-1}$ and a CDW biomass of 20.14 g $L^{-1}$ (Fig. 4). In another low shear bioreactor (centrifugal impeller bioreactor) and also under elicitation conditions of MeJA, Zhong and Zhang [46] achieved a high biomass and ginsenoside production in cell cultures of *Panax notoginseng*.

Taken as a whole, the results demonstrate the high capacity of *S. khuzistanica* cultures for producing RA, which accumulates mainly inside the cells, and the effectiveness of the MeJA elicitor treatment for increasing the production, therefore suggesting the process is suitable for scaling up to reactor level in batch mode. Scaling up to a wave-mixed BIOSTAR RM 20/50 improved both the biomass and RA production of the system, thus confirming the suitability of the wave-mixed bioreactor for the culture of *S. khuzistanica* cells. The achievement of a maximum biomass of 403.24 g $L^{-1}$ (CFW) and an RA production of 3.6 g $L^{-1}$ opens the possibility of implementing a biotechnological platform based on elicited *S. khuzistanica* cell cultures for the commercial production of the bioactive compound RA, especially considering that the percentage of RA in the studied cell line, when cultured in optimum conditions, was more than 14-fold higher than in wild plants (1.8% DW).

## Practical application

This work deals with the development of green cell factories for the production of valuable plant secondary metabolites, specifically rosmarinic acid (RA), a compound with promising biological activities for the treatment of cancer or Alzheimer's disease, among other health problems. The study shows the feasibility of using *Satureja khuzistanica* cell cultures for the biotechnological production of RA, confirming the role of methyl jasmonate as a suitable elicitor for enhancing RA yield. Furthermore, the process was successfully scaled up to a wave-mixed bioreactor without a decrease in productivity. Therefore, these results open the possibility of implementing a biotechnological platform based on elicited *S. khuzistanica* cell cultures for the production of RA on a commercial scale.

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